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Tolerogenic dendritic cells generated with dexamethasone and vitamin D3 regulate rheumatoid arthritis CD4⁺ T-cells partly via TGF- β 1

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Abbreviations:

AuToDeCRA – autologous tolerogenic dendritic cells in RA

CIA - collagen-induced arthritis

DC – dendritic cells

Dex - dexamethasone

FoxP3 - forkhead box P3

HC - healthy controls

LAP – latency-associated peptide

LPS - lipopolysaccharide

matDC - mature LPS-activated dendritic cells

PB – peripheral blood

PBMC - peripheral blood mononuclear cells

pSmad2/3 - Smad2 (pS465/pS467)/Smad3 (pS423/pS425)

RA - rheumatoid arthritis

SF – synovial fluid

SFMC - synovial fluid mononuclear cells

TGF- β 1 – transforming growth factor beta 1

TGF- β RI - transforming growth factor beta receptor 1

TGF- β RII - transforming growth factor beta receptor 2

Tmat – T-cells primed by matDC

Ttol – T-cells primed by tolDC

tolDC - tolerogenic dendritic cells

VitD3 – vitamin D3

Summary

Tolerogenic dendritic cells (tolDC) are a new immunotherapeutic tool for the treatment of rheumatoid arthritis (RA) and other autoimmune disorders. We have established a method to generate stable tolDC by pharmacological modulation of human monocyte-derived DC. These tolDC exert potent pro-tolerogenic actions on CD4⁺ T-cells. Lack of IL-12p70 production is a key immunoregulatory attribute of tolDC but does not fully explain their action. Here we show that tolDC express TGF- β 1 at both mRNA and protein level, and that expression of this immunoregulatory cytokine is significantly higher in tolDC than in mature monocyte-derived DC. By inhibiting TGF- β 1 signalling we demonstrate that tolDC regulate CD4⁺ T-cell responses in a manner that is at least partly dependent on this cytokine. Crucially, we also show that while there is no significant difference in expression of TGF β RII on CD4⁺ T-cells from RA patients and healthy controls RA patient CD4⁺ T-cells are measurably less responsive to TGF- β 1 than healthy control CD4⁺ T-cells (reduced TGF- β -induced Smad2/3 phosphorylation, FoxP3 expression and suppression of IFN- γ secretion). However, CD4⁺ T cells from RA patients can nonetheless be efficiently regulated by tolDC in a TGF- β 1-dependent manner. This work is important for the design and development of future studies investigating the potential use of tolDC as a novel immunotherapy for the treatment of RA.

Introduction

Rheumatoid arthritis (RA) is a debilitating, chronic autoimmune disease resulting in joint inflammation and progressive joint destruction. It is thought that RA arises from a breakdown of immunological self-tolerance leading to aberrant immune responses to autoantigens. CD4⁺ T-cells, specifically Th1 and Th17 cells, are thought to be the main contributors to this response via the production of proinflammatory cytokines, such as IFN- γ and IL-17A(1). There is currently no known cure for RA and current treatments involve chronic, non-antigen-specific global immunosuppression, which can be associated with numerous side effects. New paradigms for the treatment of RA are focussed on the reinstatement of self-tolerance in an antigen-specific manner, leaving immunogenic responses to pathogen-derived antigens and cancer immunity intact.

We have developed a novel, immunotherapeutic tool for inhibiting T-cell-mediated pathology, namely tolerogenic dendritic cells (tolDC)([2-6](#)). These cells are generated by treating monocyte-derived DC with the immunosuppressive glucocorticoid, dexamethasone (Dex); the vitamin D receptor agonist, vitamin D3 (VitD3); and the toll-like receptor 4 ligand, lipopolysaccharide (LPS). The phenotype of these cells is stable and is characterised by intermediate levels of the T-cell co-stimulatory molecules, CD80 and CD86, low levels of the proinflammatory cytokines, IL-12 and TNF- α , and high levels of the anti-inflammatory cytokine, IL-10. We have demonstrated that equivalent murine

tolDC can inhibit the destructive immune response in a collagen-induced arthritis (CIA) mouse model of RA(5), and we have recently completed a phase I clinical trial demonstrating the safety and feasibility of autologous tolDC therapy in inflammatory arthritis patients(7). tolDC have low immunostimulatory capacity for CD4⁺ T-cells. Specifically they skew naïve CD4⁺ T-cells responses to an anti-inflammatory profile, while they render memory CD4⁺ T-cells hyporesponsive(2-4). We have previously demonstrated that, despite the production of high levels of IL-10 by tolDC, this cytokine appears not to be essential for their regulatory action on CD4⁺ T cells, whereas deficient IL-12p70 production is essential(2).

TGF- β is a pleiotropic cytokine, produced by a wide variety of cells. Its most immunologically relevant and potent isoform is TGF- β 1, which plays an important role in immunoregulation via direct suppression of T-cell proliferation and cytokine production(8) and the induction of FoxP3⁺ Tregs(9). TGF- β 1 is produced in a latent inactive form through non-covalent linkage to latency-associated peptide (LAP)(10). LAP(TGF- β 1) can bind to the surface of cells, components of the extracellular matrix, or can exist in a soluble form. LAP(TGF- β 1) is activated (released from LAP) by a number of mechanisms, such as cleavage by thrombospondin. Activated TGF- β 1 binds to TGF- β receptor II (TGF- β RII) followed by recruitment and activation of the primary signal transducer, TGF- β RI (ALK5). This leads to phosphorylation of the receptor-regulated Smads, Smad2 and Smad3, that regulate target gene

transcription(11). Negative feedback of the pathway is mediated by the inhibitory Smad, Smad7, which can either block or degrade TGF- β RI (12).

TGF- β 1 is abundant in the affected joints of RA patients(13-19) and increases with disease duration(20). However, this high level of TGF- β 1 does not adequately regulate the pathogenic T-cell response in RA. This may be due to the overall proinflammatory cytokine environment in the synovium and the high level of T-cell activation, which are known to downregulate TGF- β RII expression(21, 22) or, alternatively, may be due to induction of inhibitory molecules, such as Smad7(23).

We aimed to determine a potential role for TGF- β 1 in the regulatory function of tolDC, and more specifically to investigate the ability of tolDC to regulate CD4⁺ T-cells from RA patients in a TGF- β 1-dependent manner. A more complete understanding of the molecular mechanisms by which tolDC regulate T-cell responses is essential for the development of future studies on tolDC immunotherapy for RA.

Materials and methods

The minimum information about tolerogenic antigen-presenting cells (MITAP) checklist was followed for the preparation of this manuscript(24). See <http://w3id.org/ontolink/mitap> for MITAP document and checklist.

Isolation of cells from peripheral blood and synovial fluid

Human samples were obtained from healthy controls (HC) and patients with RA (disease duration over one year) with informed consent and following a favorable ethical opinion from South West 3 Research Ethics Committee. All samples were transported at room temperature and were processed within 3 h of sample retrieval. Peripheral blood mononuclear cells (PBMC) were isolated from 18 ml fresh EDTA anti-coagulated peripheral blood (PB) or 5 ml Leukocyte Reduction System cones by density centrifugation on Lymphoprep (Axis-Shield Diagnostics, Dundee, UK). Synovial fluid mononuclear cells (SFMC) were isolated from 10-50 ml synovial fluid (SF) by incubation with heparin (1 U/ml) and hyaluronidase (10 U/ml) for 30 minutes at 37°C before density centrifugation on Lymphoprep. CD14⁺ monocytes were isolated from HC PBMC by positive magnetic selection using anti-CD14 magnetic microbeads and a VarioMACS (both Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T-cells were isolated from HC or RA PBMC with anti-CD4 magnetic microbeads (Miltenyi Biotec) or with RosetteSep CD4⁺ T-cell enrichment kit (StemCell Technologies, Vancouver, Canada). Purities of CD14⁺ monocytes and CD4⁺ T-cells were routinely >90%, as determined by flow cytometry (data not shown).

Generation of DC populations

Freshly isolated monocytes were immediately cultured in RPMI-1640 (Sigma, Poole, UK) supplemented with 10% FCS (Lonza, Basel, Switzerland), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma) at 0.5×10^6 cells/ml in a 1 ml volume in a 24-well culture plate (costar, Corning, NY, USA) in the presence of IL-4 and GM-CSF (50 ng/ml each, Immunotools, Friesoythe, Germany) for 7 days at 37°C with 5% CO₂. Fresh pre-warmed medium and cytokines were added on day 3. Mature DC (matDC) were matured on day 6 by addition of LPS from *Escherichia coli* (0.1 µg/ml, Sigma) for 24 h. tolDC were generated as above but with by addition of Dex (1×10^{-6} M, Sigma) at day 3 and Dex (1×10^{-6} M), the active form of vitD3, 1α,25-dihydroxyvitamin D3, (1×10^{-10} M, Leo-Pharma, Ballerup, Denmark) and LPS (0.1 µg/ml) at day 6 for 24 h. On day 7 tolDC and matDC morphology was checked using an inverted microscope – tolDC were slightly elongated and adhered to the culture plates, whereas matDC were more rounded with visible dendrites and did not adhere to the culture plates. All DC populations were extensively washed before using them in functional assays. DC phenotype was checked using flow cytometry and was consistent with tolDC exhibiting a semi-mature phenotype, expressing low levels of CD83, intermediate levels of CD80 and CD86 and high levels of HLA-DR (data not shown).

Micro Fluidic Cards

RNA was extracted from DC using an RNeasy kit (Qiagen, Crawley, UK). RNA was reverse-transcribed to cDNA using random hexamers and SuperScript II

RT (Invitrogen, Paisley, UK). cDNA samples were run on a custom Micro Fluidic Card (Applied Biosystems, Foster City, CA, USA) using an ABI Prism 7900HT system (Applied Biosystems). TGF- β 1 mRNA expression was normalised to that of human GAPDH by subtracting the comparative threshold (C_T) value of GAPDH from the C_T value of TGF- β 1 (ΔC_T). Results are expressed as $2^{-\Delta C_T}$.

Flow cytometry

Anti-human LAP(TGF- β 1)-PE antibody (27232; R&D Systems, Abingdon, UK) was used for cell surface marker analysis of DC. Anti-human CD3-APC (HIT3a; BD Bioscience, San Jose, CA, USA), CD4-FITC (RPA-T4; eBioscience Ltd., Hatfield, UK), and TGF β RII-PE (25508; R&D Systems) antibodies were used for cell surface marker analysis of PBMC and SFMC. Briefly, cells were centrifuged and resuspended in FACS buffer (PBS (Lonza) supplemented with 0.5% BSA (Sigma), 1mM EDTA (Fisher Scientific, Fair Lawn, New York, NY, USA), and 0.01% sodium azide (Sigma)). 4 μ g/ml human IgG (Grifols, Los Angeles, CA, USA), was added with antibodies to prevent Fc receptor binding. Cells were incubated on ice for 30 min, centrifuged and resuspended in FACS buffer. Intracellular FoxP3 was detected using a FoxP3-APC staining kit (PCH101; eBioscience).

Intracellular pSmad2/3 was detected using a Phosflow assay by serum starving PBMC overnight by culture in serum-free X-VIVO 15 (Lonza) at 37°C with 5% CO₂. PBMC were stimulated with 10 ng/ml TGF- β 1 (PeproTech, EC Ltd., London, UK) for 30 minutes at 37°C. Untreated control samples were set-up in

parallel. PBMC were fixed using 1X BD Phosflow Lyse/Fix Buffer (BD Bioscience) and then permeabilised using BD Perm Buffer III (BD Bioscience). To reduce background staining the cells were blocked with 2% mouse serum (Sigma) for 15 min prior to addition of anti-human CD3-Pacific Blue (UCHT-1; BD Bioscience), Smad2 (pS465/pS467)/Smad3 (pS423/pS425)-PE (pSmad2/3; O72-670; BD Bioscience) and CD4-APC-eFluor780 (SK3; eBioscience) antibodies. PBMC were incubated at room temperature for 1 h, centrifuged and resuspend in stain buffer (PBS with Ca^{2+} and Mg^{2+} (Lonza) supplemented with 0.2% BSA and 0.09% sodium azide). Data was collected on a BD FACSCanto II (BD Biosciences) and analysed using FlowJo (Tree Star Inc.). Results are shown as either the median fluorescent intensity (MFI) of the marker of interest or as a percentage of cells expressing the marker of interest.

Stimulation of cells by $\alpha\text{CD3}\alpha\text{CD28}$ expander beads and TGF- β 1

PBMC, SFMC and CD4^+ T-cells were stimulated with $\alpha\text{CD3}\alpha\text{CD28}$ expander beads (10:1 ratio; Dyna, Invitrogen) in the absence or presence of 10 ng/ml TGF- β 1 in RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Supernatants were harvested after 3 days and assayed for IFN- γ by sandwich ELISA (BD Bioscience). Percentage suppression was calculated as follows: $([\text{amount of cytokine in absence of TGF-}\beta - \text{amount of cytokine in presence of TGF-}\beta] / \text{amount of cytokine in absence of TGF-}\beta) \times 100$. The percentage of $\text{CD4}^+\text{FoxP3}^+$ cells was determined by flow cytometry.

DC-T-cell co-cultures

1x10⁴ DC were cultured with 1x10⁵ allogeneic CD4⁺ T-cells (1:10 ratio) in 200µl serum-free X-VIVO 15 medium (Lonza). TGFβRI (ALK5) inhibitor (SB-505124; Sigma) or recombinant human LAP (R&D Systems) was added where indicated. Supernatants were harvested after 6 days and assayed for IFN-γ and IL-17A by sandwich ELISA and other cytokines by an immunoassay (MSD, Maryland, USA). Proliferation was assessed by incorporation of ³H-thymidine for the last 8 h of culture by scintillation counting (Microbeta TriLux, Perkin Elmer, USA).

T-cell restimulation

Allogeneic CD4⁺ T-cells were primed with DC (1:10) in the presence or absence of 1µM SB-505124 for 6 days and rested for 4 days with 10 IU/ml IL-2 (Proleukin; Novartis Pharmaceuticals UK Ltd., Surrey, UK). No residual DC were observed in the T-cell lines. Primed T-cells were washed and restimulated with matDC from the original DC donor (1:10) in serum-free X-VIVO 15 medium (Lonza). Supernatants were harvested after 72 h and assayed for IL-10 (BD Bioscience) and IFN-γ by sandwich ELISA. Cultures were pulsed with ³H-thymidine for a further 8 hours to determine proliferation.

Statistics

Wilcoxon signed rank tests or Mann Whitney tests were performed using Prism 4.0 (GraphPad Software). All p values are two-tailed.

Results

tolDC express TGF- β 1

To investigate the role of TGF- β 1 in tolDC function we began by measuring the expression of TGF- β 1 in tolDC and matDC populations. tolDC expressed more *TGFB1* mRNA than matDC (Figure 1A). To confirm that *TGFB1* transcripts were translated, we used flow cytometry to assess TGF- β 1 protein expression on the cell surface using an anti-LAP(TGF- β 1) antibody. Mirroring the transcript data, LAP(TGF- β 1) was expressed at a higher level on the surface of tolDC as compared to matDC (Figure 1B). Together, these data show that tolDC express a higher level of TGF- β 1 than matDC at both mRNA and protein level.

TGF- β 1 is involved in tolDC regulatory function

We next determined the role of TGF- β 1 in the regulatory action of tolDC. Allogeneic HC CD4⁺ T-cells were co-cultured with DC in the presence or absence of SB-505124, a small molecule inhibitor of TGF- β RI. In keeping with our previous finding(4), tolDC showed a reduced ability to induce T-cell proliferation and IFN- γ production in CD4⁺ T-cells as compared with matDC (Figure 2A-B). Importantly, addition of SB-505124 had no effect on T-cell proliferation but enhanced the production of IFN- γ by tolDC-primed T-cells in a dose-dependent manner - restoring levels to those produced by control cells (matDC-primed T cells)(Figure 2A-B). The effect of blocking TGF- β 1 signalling in a primary MLR using 1 μ M SB-505124 results in a 2.7 fold increase in IFN- γ production from matDC-CD4⁺ T cell cultures (data not shown) and a 46.5 fold

increase in IFN- γ production from tolDC-CD4⁺ T cell cultures (summary of data shown in Figure 2B). Blocking TGF- β signalling with SB-505124 also enhanced IL-4 and IL-10 cytokine production, with minimal effect on IL-2, TNF- α , IL-17A, IL-6, IL-1- β , IL-8 and IL-13 production, by tolDC-primed T-cells, although this was not as pronounced as the effect seen for IFN- γ production (Figure 2C). We repeated the experiment using a different TGF- β signalling inhibitor, recombinant LAP, which binds and sequesters active TGF- β 1. Although LAP was less potent, similar effects were demonstrated (Figure 2D). The effects of blocking TGF- β signalling with SB-505124 were also observed at a lower DC:T cell ratio of 1:20 and 1:40 (Supplementary Figure 1).

We further investigated whether blockade of the TGF- β signalling pathway could overcome the regulatory effects of tolDC on CD4⁺ T-cells that are evident on T cell restimulation. As we have shown previously(4), HC CD4⁺ T-cells primed by tolDC (Ttol) have significantly reduced cytokine production upon restimulation as compared to matDC-primed CD4⁺ T-cells (Tmat) (Figure 2E). Importantly, addition of 1 μ M SB-505124 during priming significantly reversed the inhibition of IFN- γ and IL-10 production by tolDC to control levels (Figure 2E and Supplementary Figure 2). These data indicate that part of the regulatory activity of tolDC, specifically the ability of tolDC to suppress cytokine production by CD4⁺ T-cells, is dependent on TGF- β 1 signalling.

HC and RA CD4⁺ T-cells express TGF- β RII but RA CD4⁺ T-cells are less responsive to TGF- β 1

Proinflammatory cytokines and high levels of T cell activation, both features of RA, can induce downregulation of TGF- β RII expression(21, 22) and induce expression of inhibitory molecules, such as Smad7(23). These may render RA T-cells resistant to regulation by TGF- β 1. To investigate this, we first determined the expression of TGF- β RII on the surface of HC T-cells from PB and RA T-cells from both PB and SF. We found no significant difference in the percentage of CD4⁺ T-cells expressing TGF- β RII between HC PB T cells and RA PB or SF T cells, although the trend was for a reduced proportion of TGF- β RII⁺ CD4⁺ T-cells in RA T cells (Figure 3A).

We next examined whether HC and RA PBMC responded differently to TGF- β 1 by testing the ability of TGF- β 1 to suppress production of IFN- γ by the cells. The extent of suppression of IFN- γ by TGF- β 1 in PBMC and SFMC from RA patients was lower than in PBMC from HC (Figure 3B and Supplementary Figure 3). We also demonstrated a trend for fewer FoxP3-expressing CD4⁺ T-cells from RA patients than from HC following stimulation of T-cells in the presence of TGF- β (Figure 3C). This suggests that PBMC and SFMC from RA patients have a reduced regulatory response to TGF- β . To further explore the ability of RA T-cells to respond to TGF- β 1 we used a Phosflow assay to measure the level of phospho-Smad2/3 (pSmad2/3) in CD4⁺ T cells following TGF- β 1 stimulation. CD4⁺ T-cells from RA PB had significantly lower pSmad2/3 levels following TGF- β 1 stimulation as compared to HC CD4⁺ T-cells from PB (Figure 3D). These data suggest that CD4⁺ T-cells from RA patients have a reduced ability to respond to TGF- β 1.

RA CD4⁺ T-cells can be regulated by tolDC in a TGF- β -dependent manner

Our work demonstrates that TGF- β 1 is involved in the regulatory function of tolDC and is responsible for inducing a hyporesponsive phenotype in CD4⁺ T-cells. As RA T-cells appear to have a reduced response to TGF- β 1 we next investigated whether these cells can be tolerised by tolDC. Similar to the findings in HC, tolDC showed a reduced ability to induce T-cell proliferation and production of IFN- γ in RA CD4⁺ T-cells as compared with matDC (Figure 4). Unlike the result seen in HC CD4⁺ T-cells (Figure 2B), addition of SB-505124 to RA CD4⁺ T-cell-tolDC co-cultures induced a small increase in T-cell proliferation, although this was not restored to the levels of the control cells (matDC-primed T cells) and the effect of SB-505124 was very subtle. Interestingly, addition of SB-505124 to these cultures did enhance the production of IFN- γ by tolDC-primed T-cells, restoring levels to those of the control cells (matDC-primed T cells). These data suggest that CD4⁺ T-cells from RA patients can be regulated by tolDC partly via a TGF- β 1 dependent mechanism. Interestingly, Smad7 was found to be expressed in RA patient CD4⁺ T-cells, however it was also expressed in CD4⁺ T-cells from a proportion of HC (Supplementary Figure 4). In addition, there was a trend for increased TGF- β RII expression on CD4⁺ T-cells cultured with tolDC (Supplementary Figure 5).

Discussion

This study focussed on the role of TGF- β in the regulatory function of tolDC. We have found that tolDC express TGF- β 1 and this cytokine is involved in their ability to regulate T-cells. TGF- β 1 expression by tolDC is an important functional characteristic for the regulation of cytokine production by T-cells and plays a role in reducing both IFN- γ and IL-10 production in tolDC-primed CD4⁺ T-cells. However, TGF- β 1 does not seem to play a major role in the inhibition of T-cell proliferation. Importantly, despite the reduced ability of CD4⁺ T-cells from RA patients to respond to TGF- β 1 these cells are still regulated by tolDC in a TGF- β 1 dependent manner.

We previously investigated the effect of blocking TGF- β 1 signalling in tolDC-T-cell co-cultures and demonstrated that the use of a TGF- β 1 neutralising antibody (1D11, R&D Systems) had no effect on the regulatory action of tolDC(2). The discrepancy between the results with the neutralising antibody and those shown here using the ALK5 inhibitor SB-505124, or LAP, may be explained by the concentration of neutralising antibody used being insufficient to inhibit the amount of TGF- β 1 produced by tolDC. In addition, FCS, which contains high levels of TGF- β 1, was added to the culture medium in the original experiments. This may have led to sequestration of the neutralising antibody, thus interfering with efforts to neutralise TGF- β 1 in those assays.

We report that TGF- β 1 has differential effects on CD4⁺ T-cell proliferation and cytokine production: there was no effect on proliferation but potent inhibition of cytokine production. TGF- β 1 can have both stimulatory and inhibitory effects on T-cell proliferation and differentiation depending on the differentiation stage of the cells and the cytokine microenvironment. It has been reported that TGF- β 1 can inhibit resting CD4⁺ T-cell proliferation and cytokine production but has no inhibitory effect on activated T-cells, due to downregulation of TGF- β RII(22). Addition of IL-10 enhanced TGF- β RII expression and restored TGF- β 1 responsiveness in activated T-cells, although we have been unable to reproduce this result (data not shown). Interestingly, tolDC increase TGF- β RII expression on CD4⁺ T-cells, although the mechanism inducing this upregulation requires further investigation. Other factors involved in the proliferative response of T-cells to TGF- β 1 include CD28 co-stimulation(25). In the absence of CD28 co-stimulation, TGF- β 1 inhibits proliferation of naïve T-cells but in the presence of CD28 co-stimulation it enhances proliferation. A similar effect of CD28 co-stimulation was not observed in memory/effector cells, as TGF- β 1 enhanced their proliferation, whether co-stimulation was present or not. Our tolDC express intermediate levels of the co-stimulatory molecules CD80 and CD86, which ligate CD28 on T-cells. Therefore, the differential effect of TGF- β 1 on CD4⁺ T-cell proliferation and cytokine production reported here could be due to a combination of the cytokine microenvironment and the co-stimulatory capacity of our tolDC.

The importance of intact TGF- β signalling in RA T-cells is demonstrated by the fact that disruption of TGF- β signalling in T-cells in a CIA murine model leads to exacerbation of arthritis, which is associated with increased Th1 cytokines, IFN- γ and TNF- α (26). We have demonstrated that RA PBMC and SFMC have a reduced ability to respond to TGF- β 1, as demonstrated by a reduced level of suppression of IFN- γ production following stimulation in the presence of TGF- β 1. This is in keeping with published literature that exogenous TGF- β 1 cannot inhibit the spontaneous cytokine production of RA synovial mononuclear cells in culture(27) and cannot reduce IFN- γ production by RA synovial T-cells(28). In addition, we have shown that stimulation of RA CD4⁺ T-cells with TGF- β 1 induces less FoxP3 expression and less pSmad2/3 induction compared to cells from HC. A similar result has been reported in RA PBMC, which had deficient TGF- β 1 signalling, evidenced by reduced levels of pSmad2/3(29).

This reduced response to TGF- β 1 does not appear to be linked to a reduced level of TGF- β RII expression. The immune environment found in RA may explain the diminished response of RA T-cells to TGF- β 1. For example, the proinflammatory cytokine TNF- α , which is found in high levels in RA PB and SF, can regulate TGF- β signalling via the induction of inhibitory Smad7(23) and via AP-1 components that interfere with Smad signalling(30). Indeed, we found expression of Smad7 in RA CD4⁺ T cells. Thus, the defective response to TGF- β 1 in these cells may be due to increased negative feedback via Smad7. Defective TGF- β signalling has also been demonstrated in T-cells from other inflammatory conditions, including systemic lupus erythematosus and Crohn's

disease (31, 32). In the latter case, defective TGF- β 1-signalling was due to expression of high levels of Smad7, which could be reversed with treatments targeting this molecule(32, 33). Whether tolDC can override the negative regulatory Smad7 loop by, for example, increasing the expression of TGF- β RII on CD4⁺ T-cells (Supplementary Figure 5) and enhancing the level of Smad2/3-mediated signalling will require further investigation.

In conclusion, tolDC regulate T-cell cytokine production via expression of TGF- β 1. Despite RA T-cells having a reduced response to TGF- β 1 regulation, tolDC are still capable of regulating the cytokine response of RA T-cells. Thus tolDC may be a promising tool for the restoration of immune tolerance in RA.

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Author contributions: AEA, JDI and CMUH conceived and designed the study. JAK advised on experimental design. AEA, DJS, OYW, MB and AMP conducted experiments and data analysis. AGP, GR and JD recruited patients. AEA wrote the manuscript and DJS, JAK, JDI and CMUH reviewed and revised the manuscript.

Conflict of interest

The authors declare no financial or commercial conflicts of interest.

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Figure legends

Figure 1: tolDC express TGF- β . (A) Expression of the TGF- β 1 gene by matDC and tolDC was measured using a custom Micro Fluidic Card (Applied Biosystems). mRNA expression was normalised to that of human GAPDH by subtracting the C_T value of the gene of interest from the C_T value of the human GAPDH gene (ΔC_T). Results are expressed as $2^{-\Delta C_T}$ for 4 independent experiments. Horizontal lines represent median values. (B) matDC and tolDC were stained with anti-human LAP(TGF- β 1) antibody and were assessed by flow cytometry. Debris and dead cells were excluded on the basis of forward-scatter and side-scatter. Left panel shows one representative plot of 10 independent donors. Right panel shows the MFI for LAP(TGF- β 1) expression. Horizontal lines represent median values. N = 10. ** p < 0.01 calculated with Wilcoxon signed rank test.

Figure 2: TGF- β 1 diminishes the ability of tolDC to stimulate HC T-cells and is involved in their regulatory function. (A-D) matDC or tolDC (1×10^4 cells/well) were co-cultured with allogeneic HC CD4⁺ T-cells (1×10^5 cells/well) in the absence or presence of increasing concentrations of SB-505124, a small molecule inhibitor of TGF- β RI (A) or 1 μ M SB-505124 (B-C) or increasing concentrations of recombinant LAP (D). Proliferation (A, B and D left panels), measured using ³H-thymidine uptake, and IFN- γ production (A, B and D right panels), measured using ELISA, were assessed at day 6. (C) Additional cytokines were detected in day 6 culture supernatants from tolDC-CD4⁺ T-cell

cultures using an ELISA (IL-17A) or MSD immunoassay (all others). The fold change in cytokine production following inhibition of TGF- β 1 signalling was calculated by: concentration of cytokine produced in the presence of 1 μ M SB-505124 \div concentration of cytokine produced in the absence of 1 μ M SB-505124. IL-17A was undetectable in one experiment. Error bars in A and D represent SEM of triplicates (proliferation) or duplicates (IFN- γ production). Horizontal lines in B and C represent median values, B left panel (proliferation) n = 7; B right panel (IFN- γ production) n = 15; C (cytokines) n = 3. (E) Allogeneic HC CD4⁺ T-cells were primed with DC (1:10) for 6 days and rested for 4 days with 10 IU/ml IL-2. T-cell lines primed by matDC (Tmat), tolDC (Ttol) and tolDC + SB-505124 (Ttol-SB) were restimulated with matDC and IFN- γ (left panel) and IL-10 (right panel) production, measured using ELISA, was assessed on day 3. Results are depicted as the percentage cytokine production of Tmat cell lines. Cytokine concentrations range: IFN- γ in Ttol = 0.9 – 20.6 ng/ml and in Ttol-SB = 4.4 – 80.7 ng/ml; IL-10 in Ttol = 0.4 – 5.1 ng/ml and in Ttol-SB = 0.9 – 31.7 ng/ml. Horizontal lines represent median values, n (IFN- γ production) = 7; n (IL-10 production) = 6. * p < 0.05 and *** p < 0.0001 calculated with Wilcoxon signed rank test. # represent significant differences (p < 0.05) between Ttol and Tmat cells.

Figure 3: HC and RA CD4⁺ T-cells express TGF- β RII but RA CD4⁺ T-cells have a reduced response to TGF- β 1 stimulation. PBMC or CD4⁺ T-cells were isolated from the PB of HC or RA patients and SFMC were isolated from SF of RA patients. (A) Cells were stained with anti-human CD3, anti-human

CD4, and anti-human TGF- β RII antibodies and were assessed by flow cytometry. Debris and dead cells were excluded on the basis of forward-scatter and side-scatter. A CD3⁺CD4⁺ gate was used to identify CD4⁺ T-cells. Representative plots of TGF- β RII expression on CD4⁺ T-cells (left panel). The gate shows the percentage of CD4⁺ T-cells expressing TGF- β RII. The percentage of CD4⁺ T-cells expressing TGF- β RII (right panel). Horizontal lines represent median values. N (HC PB) = 21, n (RA PB) = 16, n (RA SF) = 5. **(B)** PBMC from HC (n=6) and PBMC (\blacktriangledown) and SFMC (\bullet) from RA patients (n=5) were stimulated with α CD3 α CD28 beads in the absence or presence of 10 ng/ml TGF- β 1 for 3 days. IFN- γ was measured by ELISA and percentage suppression was calculated as follows: $[(\text{cytokine in absence of TGF-}\beta - \text{cytokine in presence of TGF-}\beta) / \text{cytokine in absence of TGF-}\beta] \times 100$. IFN- γ concentrations range in cultures without TGF- β 1: HC = 1.1 – 32.4 ng/ml and RA patients = 0.7 – 32.2 ng/ml; and in cultures with TGF- β 1: HC = 0.1 – 5.3 ng/ml and RA patients = 0.5 – 79.1 ng/ml. **(C)** CD4⁺ T-cells from HC PB (n=6) and RA patient PB (n=4) were stimulated with α CD3 α CD28 beads in the presence of 10 ng/ml TGF- β 1 for 3 days. The percentage of CD4⁺FoxP3⁺ was determined by flow cytometry. **(D)** PBMC were left unstimulated or were stimulated with 10 ng/ml TGF- β 1 for 30 minutes before being fixed and permeabilised. PBMC were blocked with 2% mouse serum and stained with anti-human CD3, anti-human CD4 and anti-human pSmad2/3 antibodies before assessment by flow cytometry. Debris and dead cells were excluded on the basis of forward-scatter and side-scatter. A CD3⁺CD4⁺ gate was used to identify CD4⁺ T-cells. Representative plots of pSmad2/3 expression on CD4⁺ T-cells in unstimulated

and stimulated conditions (left panel). Fold induction of pSmad2/3 in CD4⁺ T-cells following TGF- β 1 stimulation calculated by dividing the MFI of stimulated cells by the MFI of unstimulated cells (right panel). Horizontal lines represent median values. N = 6. * p < 0.05 and ** p < 0.01 calculated using a Mann Whitney test.

Figure 4: RA CD4⁺ T-cells can be regulated by tolDC in a TGF- β -dependent manner. CD4⁺ T-cells (1×10^5 cells/well) from RA patients were co-cultured with allogenic matDC or tolDC (1×10^4 cells/well) from a healthy control in the absence or presence of 1 μ M SB-505124, a small molecule inhibitor of TGF- β RI. Proliferation (left panel), measured using ³H-thymidine uptake, and IFN- γ production (right panel), measured using ELISA, were assessed at day 6. N = 18. Horizontal lines represent median values. ** p < 0.01 and *** p < 0.0005 calculated with Wilcoxon signed rank test.

Figure 1

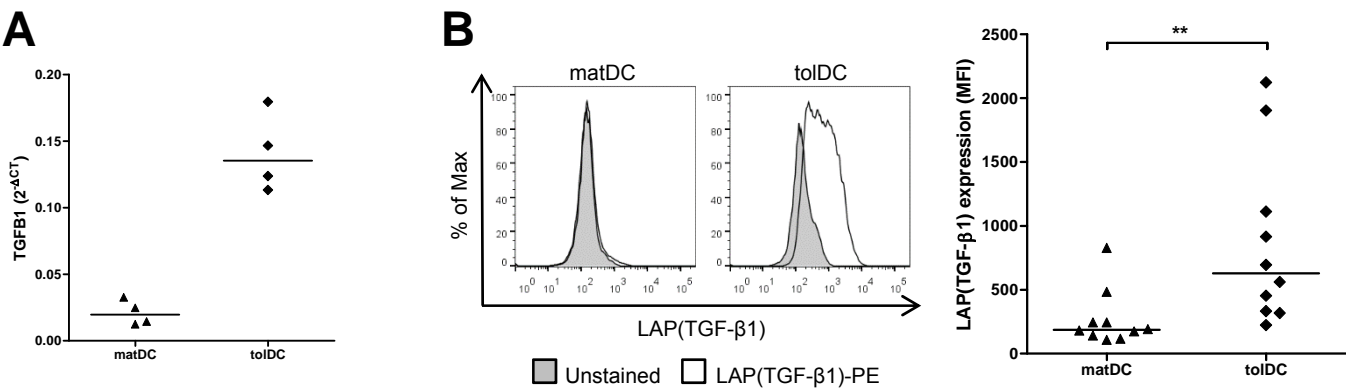
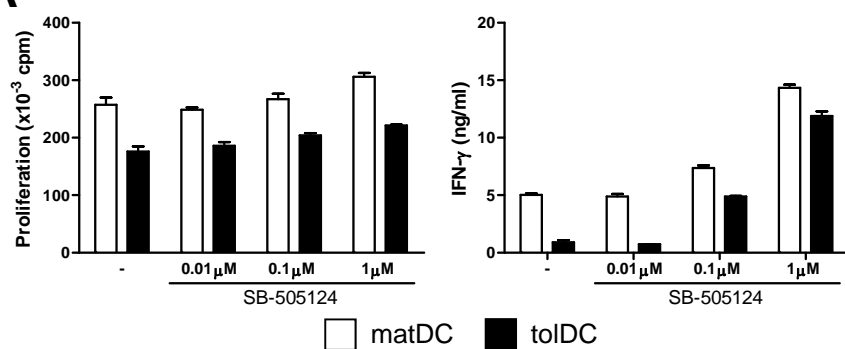
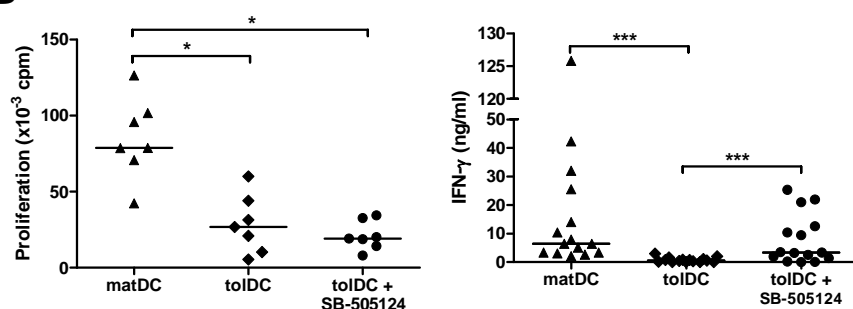


Figure 2

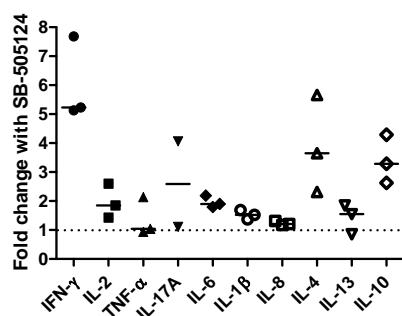
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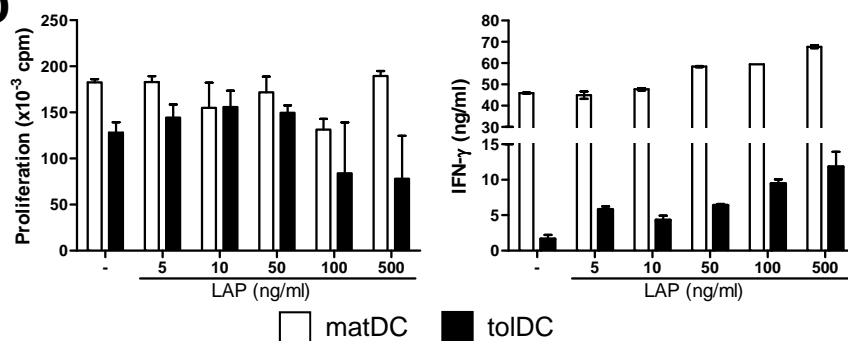
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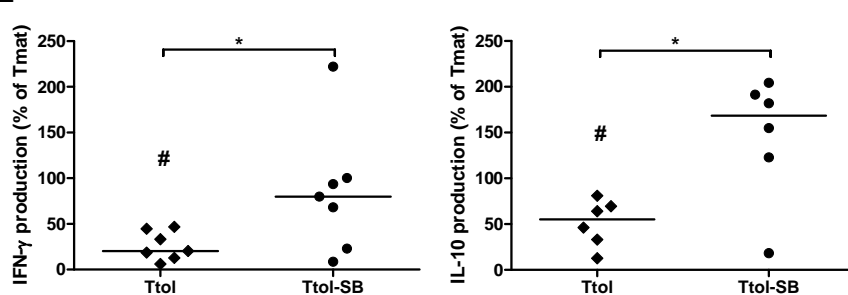
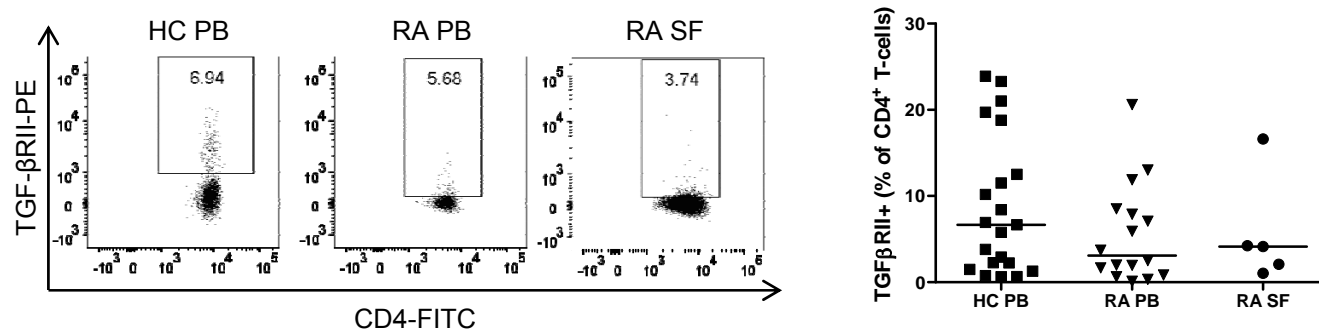
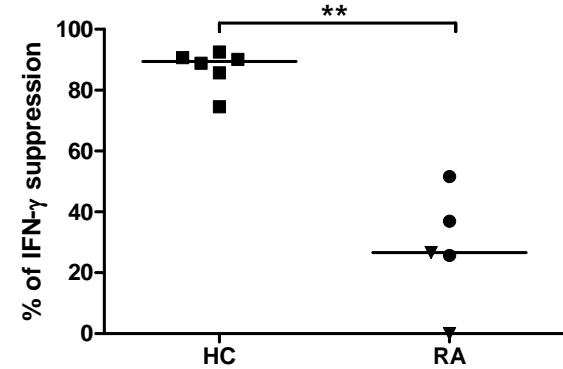


Figure 3

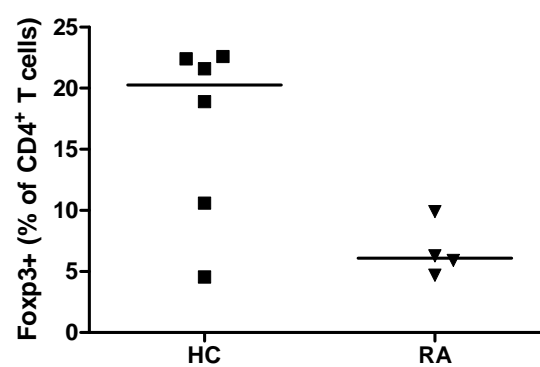
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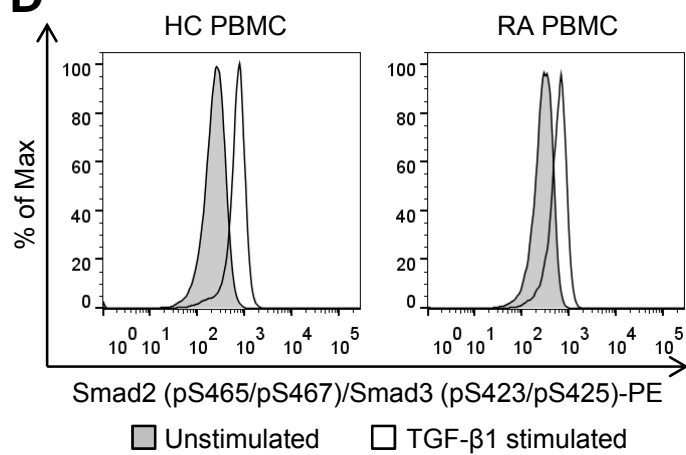
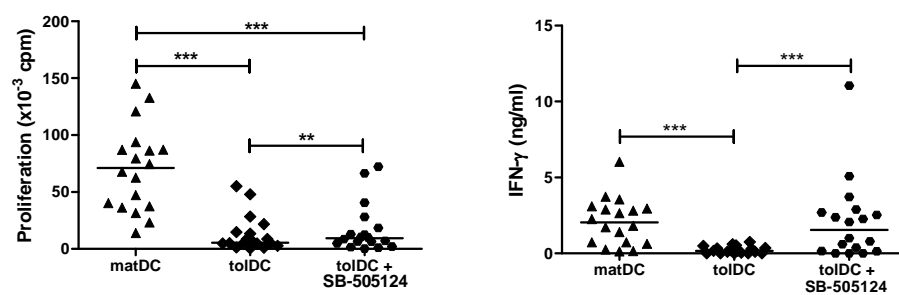
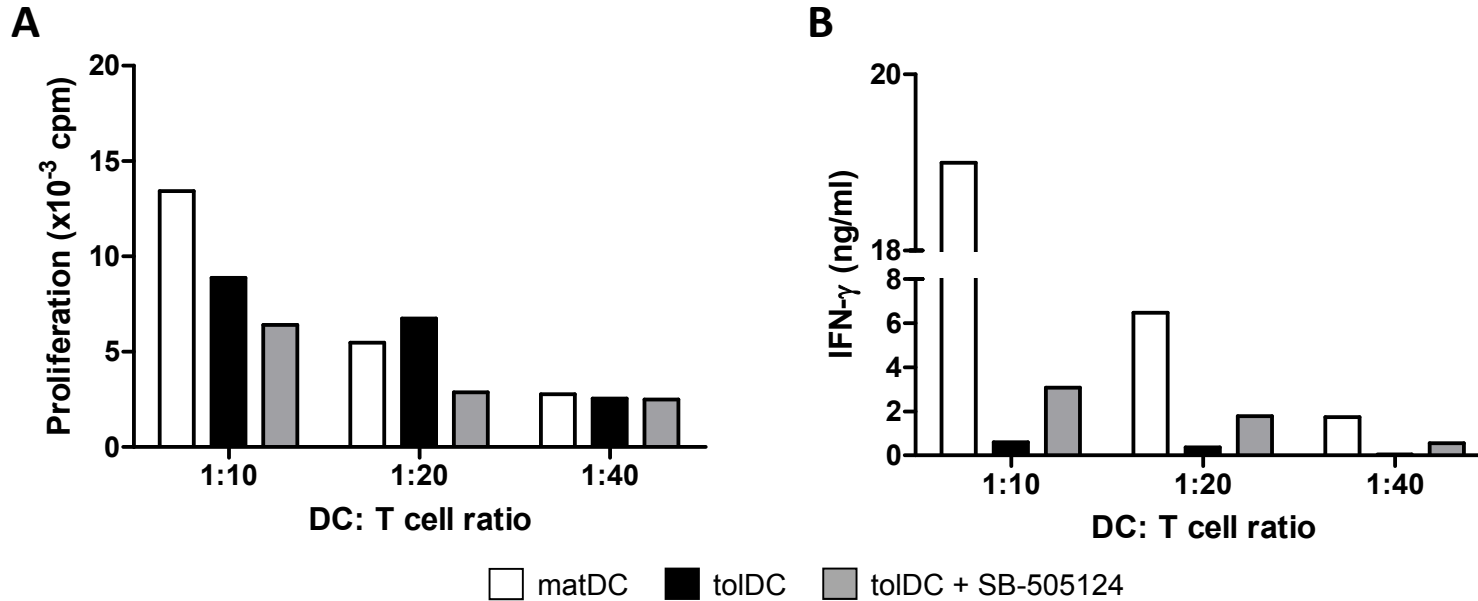
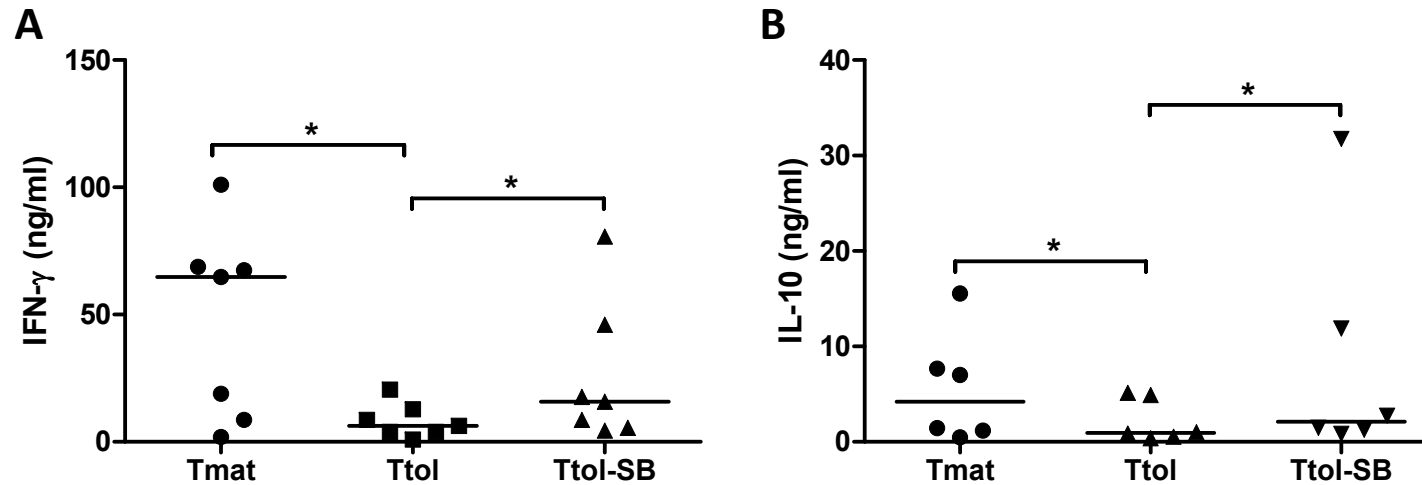


Figure 4

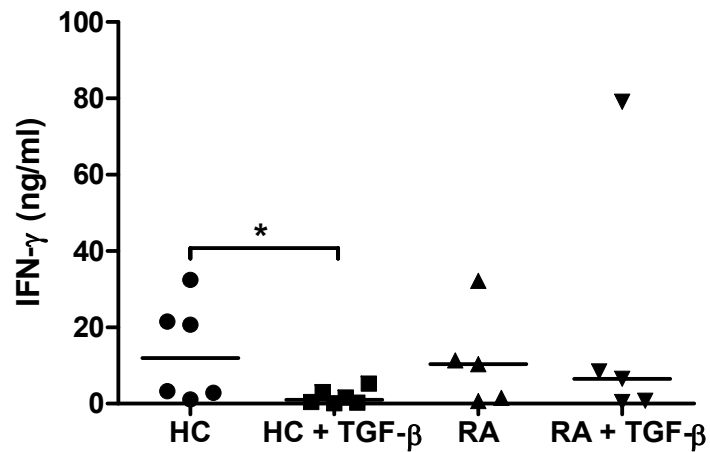




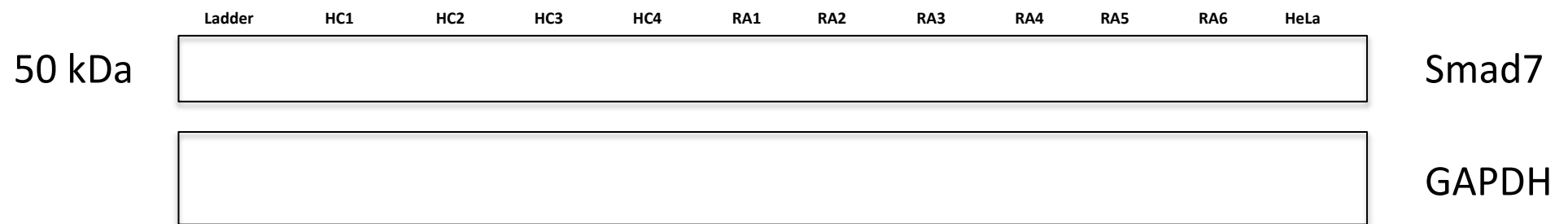
Supplementary Figure 1: TGF- β 1 diminishes the ability of tolDC to stimulate HC T-cells at different DC: T cell ratios. matDC or tolDC were co-cultured with allogeneic HC CD4⁺ T-cells (1x10⁵ cells/well) at different DC: T cell ratios in the absence or presence of 1 μ M SB-505124, a small molecule inhibitor of TGF- β RI. Proliferation (**A**), measured using ³H-thymidine uptake, and IFN- γ production (**B**), measured using a MSD immunoassay, were assessed at day 6. Columns represent median values for n = 3.



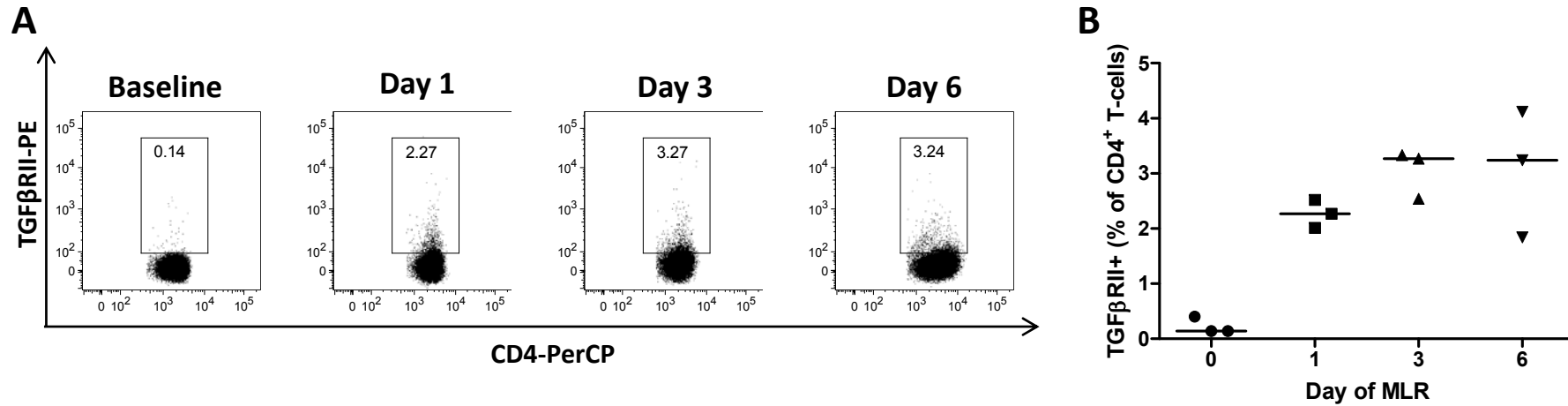
Supplementary Figure 2: Cytokine concentrations in restimulation assays. Allogeneic HC CD4⁺ T-cells were primed with DC (1:10) for 6 days and rested for 4 days with 10 IU/ml IL-2. T-cell lines primed by matDC (Tmat), tolDC (Ttol) and tolDC + SB-505124 (Ttol-SB) were restimulated with matDC and IFN- γ (**A**) and IL-10 (**B**) production, measured using ELISA, was assessed on day 3. Horizontal lines represent median values, n (IFN- γ production) = 7; n (IL-10 production) = 6. * $p < 0.05$ calculated with Wilcoxon signed rank test. Figure 2E shows these results depicted as the percentage cytokine production of Tmat cell lines.



Supplementary Figure 3: RA CD4⁺ T-cells have a reduced response to TGF-β1 stimulation. PBMC from HC (n=6) and PBMC and SFMC from RA patients (n=5) were stimulated with αCD3αCD28 beads in the absence or presence of 10 ng/ml TGF-β1 for 3 days. IFN-γ was measured by ELISA. Horizontal lines represent median values. * p < 0.05 calculated using a Wilcoxon signed rank test. Figure 3B shows these results depicted as the percentage suppression of cultures containing TGF-β1 compared to those without.



Supplementary Figure 4: RA CD4⁺ T-cells express Smad7. CD4⁺ T-cells from HC (n=4) and RA patients (n=6) were isolated and lysed in RIPA buffer before analysis using western blot. Blots were probed with anti-human Smad7 (clone 293039; R&D systems – MW 50KDa) and anti-human GAPDH (clone D16H11; Cell Signalling – MW 37KDa). HeLa cell lysate was used as a positive control.



Supplementary Figure 5: tolDC induce TGFβRII expression on T-cells. CD4⁺ T-cells (1x10⁶ cells/well) were co-cultured with allogeneic tolDC (1x10⁵ cells/well) for 1 day, 3 days or 6 days. Cells were stained with anti-human CD3, anti-human CD4, and anti-human TGF-βRII antibodies and were assessed by flow cytometry. Debris and dead cells were excluded on the basis of forward-scatter and side-scatter. A CD3⁺CD4⁺ gate was used to identify CD4⁺ T-cells. **(A)** Representative plots of TGF-βRII expression on CD4⁺ T-cells. The gate shows the percentage of CD4⁺ T-cells expressing TGF-βRII. **(B)** The percentage of CD4⁺ T-cells expressing TGF-βRII at the different time-points. Bars represent median values for n = 3.